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Dystrophin gene analysis in Duchenne/Becker dystrophy in a Malaysian population using multiplex polymerase chain reaction

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Abstract

Dystrophinopathy is the commonest form of muscular dystrophy and comprises clinically recognized forms, Duchenne dystrophy and Becker dystrophy. Mutations in the dystrophin gene which consist of large gene deletions (65%), duplications (5%) and point mutations (30%) are responsible for reducing the amount of functional dystrophin protein in skeletal muscle fibres leading to fibre destruction and disease. The aims of this study are to investigate the detection rate, types and distribution of large gene deletions in Malaysian dystrophinopathy patients using the multiplex polymerase chain reaction (MPCR). MPCR of 18 "hot-spot deletion" regions along the dystrophin gene was performed on DNA from 48 muscle biopsy-confirmed cases of dystrophinopathy. A positive detection rate of 58% (28/48) was observed, where 84% (16/19) Indian, 35% (6/17) Chinese and 50% (6/12) Malay ethnic groups showed deletions in their dystrophin genes. The Malaysian Indians appear to have a higher prevalence for large gene deletions compared to the Chinese and Malays. Further analyses of 42 confirmed positive cases (present 28 plus previous 14 cases) by MPCR showed the majority of deletions were in the mid-distal region of the dystrophin gene (81% in exons 45-60). The MPCR is a specific and sensitive method for confirmation of gene deletions responsible for dystrophinopathy.

INTRODUCTION

The commonest type of muscular dystrophy is dystrophinopathy that includes the clinically more severe form of Duchenne dystrophy (DD) and the less severe form, Becker dystrophy (BD).[1] Mutations in the large dystrophin gene that consists of 79 exons, result in reduced or absent functional dystrophin protein in skeletal muscles, leading to varying clinical severity. About 65% of DD/BD cases are the result of large scale gene deletions[2], while about 5% are due to duplications[3], with the remaining 30% arising from point mutations.[4]

Sequencing the dystrophin gene is time consuming because of the complex organisation of the introns and exons that spans 2.4 million base pairs (Mbp). However, it is fortunate that many of the large gene deletions within the dystrophin gene can be detected in specific "hotspot areas" of the gene. These "hotspots" are clustered in two main regions - at the 5' proximal portion of the gene (exons 1, 3, 4, 5, 8, 13, 19) and within the mid-distal region (exons 42 - 45, 47, 48, 50 - 53, 60).[2,5,6] DNA amplification by the multiplex polymerase chain reaction (MPCR) can detect 98% of the large scale deletions in the dystrophin gene.[5]

The MPCR differs from the standard PCR in that more than two primer sets are used to amplify different regions of target DNA in a single reaction. The advantage of MPCR is its rapidity as multiple target sequences are amplified simultaneously. MPCR requires only a very small amount of DNA that can be extracted from chorionic villi, blood and muscle samples.

The main objective of this study is to determine the detection rate of large gene deletions in Malaysian DD/BD patients using an established in-house MPCR technique. In addition, the multiracial composition of the Malaysian population could possibly lend itself to studying racial differences in dystrophinopathy, if any. There are currently no published reports of molecular analysis of the dystrophin gene in the Malaysian population.

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METHODS

Patient samples and DNA extraction

Forty-eight cases of dystrophinopathy confirmed by muscle biopsy and dystrophin protein immunohistochemistry were studied using MPCR. DNA from these cases was extracted from 19 blood and 29 frozen muscle samples. Blood samples were collected in EDTA tubes and stored at -20°C for DNA extraction. Frozen muscle samples were retrieved from the archives of the Dept of Pathology, Faculty of Medicine, University of Malaya. From each frozen muscle sample, a small piece (3mm x 3mm) was cut out in a cold -20°C cryostat and care was taken to avoid external DNA contamination by changing new blades, forceps and autoclaved aluminium foils on the cutting board between samples. Archived muscle DNA were used for the 29 cases because either no blood was available for DNA analysis or the DNA extracted from blood samples were in such poor condition that it did not allow for optimal DNA amplification. DNA was extracted from both muscle and blood using the proteinase K/phenol-chloroform extraction techniques. Extracted DNA was solubilised in water and stored at -70°C. Thus, a total of 48 patient (19 Indians, 17 Chinese and 12 Malays) known cases of DD/BD were used to study the detection rate of dystrophinopathy using MPCR. Ethical consent was obtained from the patients or parents for molecular characterisation of the dystrophin gene.

Multiplex polymerase chain reaction (MPCR)

MPCR was carried out using 25-50 ng/ml of extracted DNA. Primers sets for amplification of the 18 exons were synthesized according to published sequences.[2,5,6] The primers were divided into 4 groups (M1, M2, M3 and M4) for 4 separate MPCR assays to allow optimum visualization of the amplified products that differed from each other by a small number of bases (Tables 1 - 4). Group M1 amplified exons 42, 45, 48, 51, 52 and 53; M2 exons 43, 44, 47, 50 and 60; M3 exons 1, 3, 4, and 13 and M4 exons 8, 6 and 19, respectively. MPCR was carried out with initial denaturation at 94°C for 5 min followed by 20 cycles of annealing at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 2 min. A final extension of 10 min was carried out at 72°C. Amplified MPCR products were electrophoresis in 2% agarose gels at 80 volts for 60 minutes. Appropriate positive DNA controls were included in every MPCR reaction and comprised DNA from a normal male and normal female. A DNA blank where no DNA was added to the MPCR reaction mixture was also included in every MPCR to

Table 1: Primer sequences used for the amplification of exons 42, 45, 48, 51, 52 and 53

Group M1 consist of 6 primer sets; the exons are arranged according to their product size from the largest to the smallest (top to bottom)

Exon	Products (bp)	Forward primer (5' to 3')	Reverse primer (3' to 5')
48	506	TTG/AAT/ACA/TTG/GTT/AAA/TCC/CAA/CAT/G	CCT/GAA/TAA/AGT/CTT/CCT/TAC/CAC/AC
51	388	GAA/ATT/GGC/TCT/TTA/GCT/TGT/GTT/TC	GGA/GAG/TAA/AGT/GAT/TGG/TGG/AAA/ATC
45	307	CTT/TCT/TTG/CCA/GTA/CAA/CTG/CAT/GTG	CAT/TCC/TAT/TAG/ATC/TGT/CGC/CCT/AC
53	212	TTG/AAA/GAA/TTC/AGA/ATC/AGT/GGG/ATG	CTT/GGT/TTC/TGT/GAT/TTT/CTT/TTG/GAT/TG
42	155	CAC/ACT/GTC/CGT/GAA/GAA/ACG/ATG/ATG	TTA/GCACAG/AGG/TCA/GGA/GCA/TTG/AG
52	113	AAT/GCA/GGA/TTT/GGA/ACA/GAG/GCG/TCC	TTC/GAT/CCG/TAA/TGA/TTG/TTC/TAG/CCT/C

Table 2: Primer sequences used for the amplification of exons 43, 44, 47, 50 and 60

Group M2 consist of 5 primer sets; the exons are arranged according to their product size from the largest to the smallest (top to bottom)

Exon	Products (bp)	Forward primer (5' to 3')	Reverse primer (3' to 5')
44	426	GTT/GTG/TGT/ACA/TCG/TAG/GTG/TGT/A	TCC/ATC/ACC/CTT/CAG/AAC/CTG/ATC/T
43	357	GAA/CAT/GTC/AAA/GTC/ACT/GGA/CTT/CAT/GG	ATA/TAT/GTG/TTA/CCT/ACC/CTT/GTC/GGT/CC
50	271	CAC/CAA/ATG/GAT/TAA/GAT/GTT/CAT/GAA/T	TCT/CTC/TCA/CCC/AGT/CAT/CAC/TTC/ATA/G
47	181	CGT/TGT/TGC/ATT/TGT/CTG/TTT/CAG/TTA/C	GTC/TAA/CCT/TTA/TCC/ACT/GGA/GAT/TTG
60	139	AGG/AGA/AAT/TGC/GCC/TCT/GAA/AGA/GAA/CG	CTG/CAG/AAG/CTT/CCA/TCT/GGT/GTT/CAG/G

monitor for DNA contamination.

In order to study the types and distribution of deletions in the dystrophin gene in the different ethnic groups in Malaysia, an additional 14 dystrophinopathy cases with previously confirmed deletions were analyzed together with results from the above 28 positive MPCR cases. Results from the additional 14 cases were from previously characterized blood DNA samples in the Department of Molecular Medicine, University of Malaya.

RESULTS

The MPCR optimized was both sensitive and specific for the confirmation of deletions in the dystrophin gene. Gel electrophoresis results showed distinct amplified MPCR products for the non-deleted gene sequences in controls and patients. (Figure 1; lanes 2 - 4 and lanes 7 - 9). In patients with gene deletions, no MPCR products for the respective deleted exon were observed (lanes 5 and 6). The DNA blanks did not show any MPCR products. Figure 1 shows the gel

Table 3: Primer sequences used for the amplification of exons 1, 3, 4 and 13

Group M3 consist of 6 primer sets; the exons are arranged according to their product size from the largest to the smallest (top to bottom)

Exon	Products (bp)	Forward primer (5' to 3')	Reverse primer (3' to 5')
1	535	GAA/GAT/CTA/GAC/AGT/GGA/TAC/AGTA/ACA/AAT/GCA/TG	TTC/TCC/GAA/GGT/AAT/TGC/CTC/CCA/GAT/CTG/AGT/CC
3	410	TCA/TCC/ATC/ATC/TTC/GGC/AGA/TTA/A	CAG/GCG/GTA/GAG/TAT/GCC/AAA/TGA/AAA/TCA
13	238	AAT/AGG/AGT/ACC/TGA/GAT/GTA/GCA/GAA/AT	CTG/ACC/TTA/AGT/TGT/TCT/TCC/AAA/GCA/G
4	196	TTG/TCG/GTC/TCC/TGC/TGG/TCA/GTG	CAA/AGC/CCT/CAC/TCA/AAC/ATG/AAG/C

Table 4: Primer sequences used for the amplification of exons 6, 8 and 19

Group M4 consist of 6 primer sets; the exons are arranged according to their product size from the largest to the smallest (top to bottom)

Exon	Products (bp)	Forward primer (5' to 3')	Reverse primer (3' to 5')
19	459	TTC/TAC/CAC/ATC/CCA/TTT/TCT/TCC/A	GAT/GGC/AAA/AGT/GTT/GAG/AAA/AAG/TC
8	360	GTC/CTT/TAC/ACA/CTT/TAC/CTG/TTG/AG	GGC/CTC/ATT/CTC/ATG/TTC/TAA/TTA/G
6	202	CCA/CAT/GTA/GGT/CAA/AAA/TGT/AAT/GAA	GTC/TCA/GTA/ATC/TTC/TTA/CCT/ATG/ACT/ATG/G

electrophoresis results after MPCR with M2 for the amplification of exons 43, 44, 47, 50 and 60 in the dystrophin gene.

Large-scale gene deletions were observed in 58% (28/48) of the cases. Out of 19 Indian patients, 16 (84%) were positive; 6 (35%) Chinese patients and 6 Malay patients (50%) were also positive for deletions in the dystrophin gene. Twenty cases (42%) showed no deletions.

To study the types and distribution of the large scale deletions present in the different ethnic groups among Malaysians, results from the above 28 MPCR positive cases from this study were analysed together with a previous group of 14 MPCR positive cases. Results from the combined 42 MPCR positive cases showed 19% (8/42) had deletions in the 5'- proximal region and 81% (34/42) showed deletions in the mid-distal 3' end of the dystrophin gene (exons 42-60) (Table 5). There is a higher frequency of deletions in the mid-distal region of the gene in all the three ethnic groups being present in 84% (16/19) of Malaysian Indians, 85% (11/13) of Chinese and 70% (7/10) of Malays studied. There were no deletions in exon 60. Figure 2 shows the distribution of gene deletions in the 18 exons in the dystrophin gene observed in the three ethnic groups.

DISCUSSION

In this study, MPCR was used to detect large-scale gene deletions in 48 muscle biopsy-confirmed cases of dystrophinopathy. A positive detection rate of 58% (28/48) in the 5'-proximal (exons 1, 3, 4, 6, 8, 13, 19) and mid-distal (exons 42, 43, 44, 45, 47, 48, 50, 51, 52, 53, 60) hotspot regions was observed. Twenty muscle biopsy

Table 5: Positive MPCR cases with detailed results of large gene deletions in the dystrophin gene in the Malaysian Indians, Chinese and Malays

Hotspot regions	Race				
	Indian n = 19	Chinese n = 13	Malay n = 10	Total (n = 42)	%
5' proximal region (exons 1, 3, 4, 6, 8, 13, 19)	3	2	3	8	19
Mid-distal region (exons 42, 43, 44. 45, 47, 48, 50, 51, 52, 53, 60)	16	11	7	34	81
Total	19	13	10	42	100

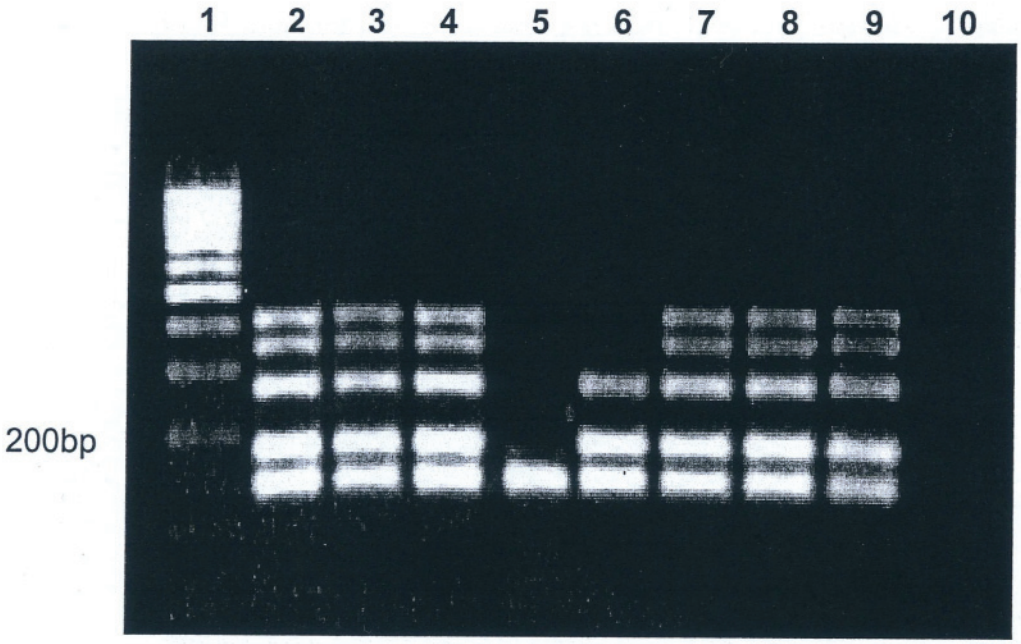

Figure 1. Gel electrophoresis results after MPCR with M2 for the amplification of exons 43, 44, 47, 50 and 60 in the dystrophin gene.

Lane 1: 100 bp molecular weight ladder; lane 2: DNA from a normal male showing no deletions (presence of exons 44 (426 bp), 43 (357 bp), 50 (271 bp), 47 (181 bp) and 60 (139 bp); lane 3: DNA from a normal female showing no deletions; lane 4: DNA from a DMD patient showing no deletions; lane 5: DNA from a DMD patient showing deletions of exons 44, 43, 50 and 47; lane 6: DNA from a DMD patient showing deletions of exons 44 and 43; lanes 7 - 9: DNA from DMD patients showing no deletions; lane 10: DNA blank where no DNA was added to the MPCR reaction.

cases showed no deletions. Positive detection rates reported in other studies using the MPCR showed a large variation ranging from 32% to 88%. In Asia, namely Arab, Chinese, and North Indian populations, the rate was 63.4%, 59.4% and 70.2% respectively.[7-9] A multinational study consisting Singaporean, Japanese and Vietnamese populations showed lower detection rates at 40%, 51%, and 32%, respectively.[10] However, this study did not use muscle biopsy confirmed diagnosis of dystrophinopathy.

Part of the reason for higher detection rates in some of the studies may be related to using a MPCR that includes more than the standard 18 hotspot exons. In the North Indian and Arab studies[7,8], the MPCR used included the amplification of additional exons - exons 20, 25, and 28. In a Venezuelan study where only 14 exons were studied, the detection rate reported was only 37 %.[11] However, in a study of a cohort of Vietnamese patients, the detection rate was only 32.4% despite a full complement of exons being amplified.[10] The detection rate obtained in our study of 58 % was found to be similar to the rate of 59.4% found in a similar study performed on a Chinese population.[9]

The frequency of MPCR-detectable large-scale deletions in Indians compared to non-Indians (Malay and Chinese) seems to be significantly higher (p= 0.003). Whether or not the Indian ethnic group has a true higher prevalence for large-scale gene deletions (as opposed to point and other mutations) needs to be confirmed in a larger series. Singh *et al*. interestingly showed that 73 % of Northern Indians had a higher prevalence for large-scale gene deletions in which 81.2 % of the deletions were found in the mid–distal region.[7] The results from this study showed that the Malaysian Indian ethnic group are consistent with their findings. It has to be pointed out however, that generally the incidence of dystrophinopathy does not vary among races.[12] It has also been suggested that due to the genetic drift, some local DNA environments may have been formed in the wild–type dystrophin gene that has allowed this locus to be predisposed to a higher frequency of breakpoint deletions, and thus a higher incidence of the disease in certain populations.[7]

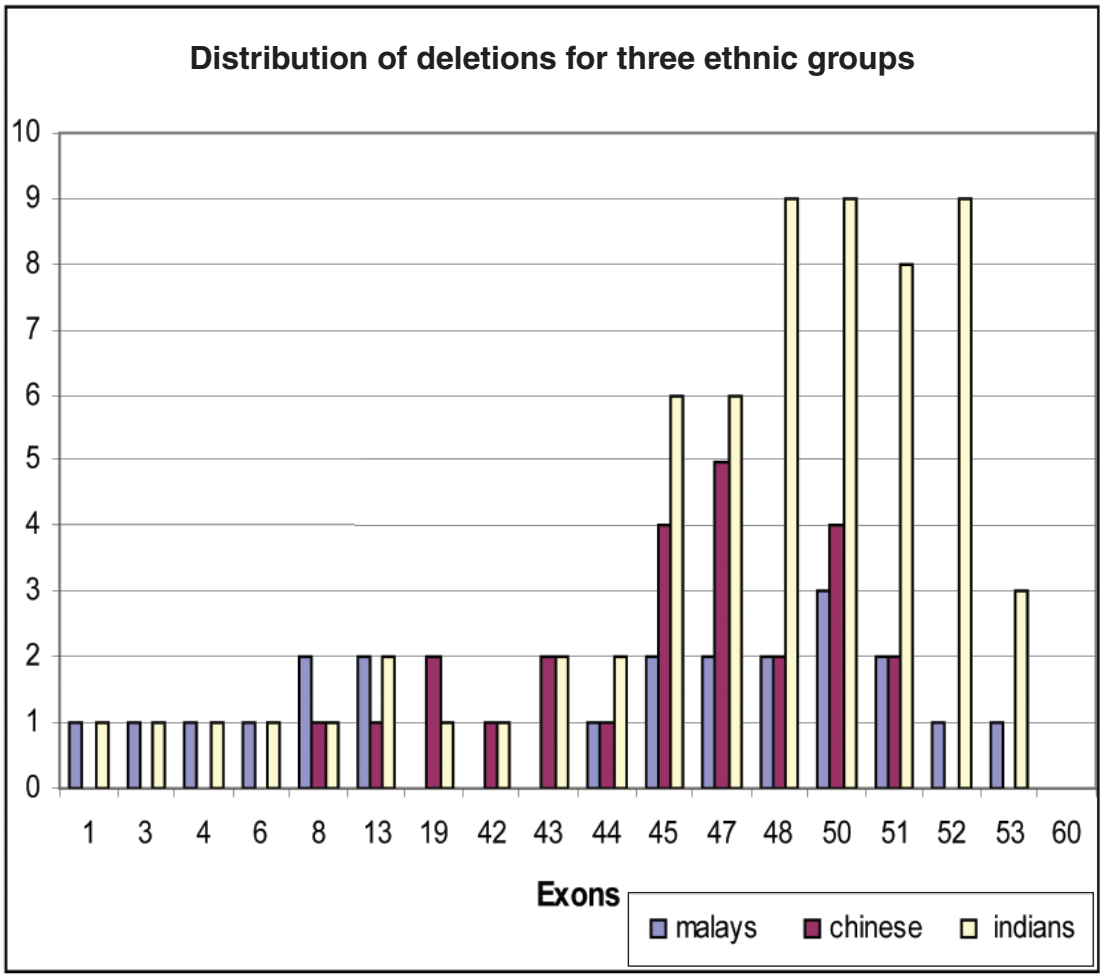

Figure 2. Bar chart showing the distribution of gene deletions in the18 exons in the dystrophin gene as observed in the Malaysian Indians, Chinese and Malays. The Y-axis represents the number of patients and there were a total of 110 patients accounting for all the deletions in the exons amplified. The reason for the larger number (study involved only 42 patients) is a result of some patients having more than one exon deleted in the dystrophin gene.

In most of the studies reported, about one third of the deletions clustered at the proximal hotspots, with the remainder were located in the mid-distal region. However, in our study, 81% of the deletions were found in the mid-distal region (Table 5). This observation was also reported in two other studies carried out in German[13] and North Indian[7] populations where deletions in the mid-distal region were 92.5 % and 81.8 %, respectively.

This study shows that the majority of dystrophinopathies can be diagnosed by MPCR, hence this should be the first line of investigation rather than the more invasive muscle biopsy. However, muscle biopsy still has a role to play in situations where MPCR is not available or showed a negative result. When sequencing of the whole dystrophin gene becomes more readily accessible and less expensive, the need for muscle biopsy should become even lesser unless dystrophin gene mutations has been excluded and other limb girdle muscular dystrophies are considered in the differential diagnosis.

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